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EFFICIENT PURIFICATION OF SOMATOMEDIN-C/INSULIN-LIKE GROWTH FACTOR I USING IMMUNOAFFINITY CHROMATOGRAPHY

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Somatomedin-C/insulin-like growth factor I was purified from human plasma using a monoclonal antibody affinity column. Combining immunoaffinity chromatography with standard protein purification methods resulted in an overall recovery of 18%. The 35 µg of somatomedin-C/insulin-like growth factor I purified from 500 ml of plasma appeared as a single band when analyzed by polyacrylamide gel electrophoresis and could be used in radioimmunoassay and receptor binding studies.

Research requiring highly purified somatomedins has been constrained by the scarcity of the pure hormones. Since isolation of somatomedin-C (also known as insulin-like growth factor I) from other plasma proteins entails a 500,000 fold purification, acquisition of substantial quantities of pure hormone from unprocessed plasma is impractical using conventional purification techniques. Using an immunoaffinity column of monoclonal anti-Sm-C/IGF-I antibody we rapidly obtained 35 µg of Sm-C/IGF-I from 500 ml of acromegalic plasma. This material was suitable for iodination and limited in vitro studies.

MATERIALS AND METHODS:

Five hundred ml of citrated plasma were obtained by plasmapheresis from an adult with untreated acromegaly and stored at -20°C. The plasma concentration of Sm-C/IGF-I determined by RIA of an acid-ethanol extract (1) was 379 ng/ml. The purification was monitored with the Sm-C/IGF-I RIA as previously described (2) using a serum standard (Ortho 1778-5; Ortho Diagnostics, Raritan, NJ) for assays to locate active fractions during purification and a highly purified preparation of Sm-C/IGF-I as the standard for

ABBREVIATIONS:
Sm-C/IGF-I, Somatomedin-C/Insulin-like growth factor I; RIA, radioimmunoassay; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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quantifying purification yield. In addition, [^{125}I]-Sm-C/IGF-I was added to the mixture and used to aid localization of the peptide during purification. Purity was assessed by analyzing partially purified material as well as the final product by SDS-PAGE. The four major steps used to isolate Sm-C/IGF-I were: 1) gel filtration in acid, 2) immunoaffinity chromatography, 3) chromatofocusing and 4) reverse-phase liquid chromatography. All purification procedures, except for liquid chromatography, were performed at 4°C.

Gel Filtration:

Preliminary experiments showed that plasma somatomedin binding proteins inhibited absorption of Sm-C/IGF-I by the immunoaffinity column. Therefore, the binding proteins first were removed by gel filtration in acid. The acromegalic plasma was dialyzed for 24 hours against 0.05 M sodium acetate, pH 3.8 in small pore dialysis tubing (Spectrapor 3; Spectrum Medical Industries, Los Angeles, CA), and then chromatographed on a 10 x 90 cm column of Sephadex G-50 (Pharmacia Inc., Piscataway, NJ) equilibrated with dialysis buffer. Fractions containing immunoreactive Sm-C/IGF-I were pooled and stored at -20°C.

Affinity Chromatography:

The monoclonal antibody for Sm-C/IGF-I was generated by hyperimmunization of a Balb mouse followed by in vitro boosting of the splenocytes by exposure to pure Sm-C/IGF-I in thymocyte conditioned media as previously reported (3). Hybridoma clone Sm 1.2 produced antibody with an affinity for Sm-C/IGF-I of 1.0 x 10^{10} M^{-1}. Insulin-like growth factor II (gift of Dr. Rene Humbel) was 5% as potent in competing with [^{125}I]-Sm-C/IGF-I for binding to Sm 1.2 and insulin displayed no crossreactivity at 10^{-6} M. This clone was amplified in the ascites fluid of pristane primed Balb mice and the antibody partially purified by sequential precipitation with 18% and 15% sodium sulfate. The antibody was coupled to CNBr-activated Sepharose 4B (Pharmacia) at a ratio of 2 mg protein per ml of hydrated gel and a portion of this product was used to construct an 11 ml affinity column. The pooled eluates from Sephadex G-50 columns were neutralized to pH 7.4 and applied to the affinity column. Following a phosphate-buffered saline (pH 7.4) wash, hormone bound to the column was eluted by a series of buffers of increasing ionic strength and decreasing pH. (details in Fig. 3).

Chromatofocusing:

Fractions from the affinity column corresponding to the [^{125}I]-Sm-C/IGF-I elution peak were lyophilized and resuspended in an aqueous solution of 10% polybuffer 96 (Pharmacia) adjusted to pH 6.0 with acetic acid. The sample was applied to a 20 ml PBE 94 (Pharmacia) column equilibrated with 0.025M ethanolamine-HCOOH, pH 9.4, and elution continued with the polybuffer solution. Fractions of pH 8.0-8.5 were acidified to pH 4.0 immediately after pH measurement and then reapplied in neutral phosphate buffered saline to the immunoaffinity column to separate Sm-C/IGF-I from the contaminating polybuffers which were not absorbed by the column. The Sm-C/IGF-I was then eluted as described above.

Reverse Phase Liquid Chromatography:

The final purification step was modified from a previously described method (4). The product of the second immunoaffinity column was applied to an octadecylsilane column (C18; Waters Associates, Milford, Mass.) in 0.1% trifluoroacetic acid and elution begun by a linear gradient of 0-31% acetonitrile in 0.1% trifluoroacetic acid over 15 min. The acetonitrile concentration was maintained at 31% until the UV absorbance (215 nm) of the eluate reached baseline after which the column elution was continued with a linear gradient of 31-60% acetonitrile.

RESULTS:

Chromatography on G-50:

When the acid dialyzed plasma was chromatographed on Sephadex G-50, immunoreactive Sm-C/IGF-I eluted after the major plasma proteins at a Kav of

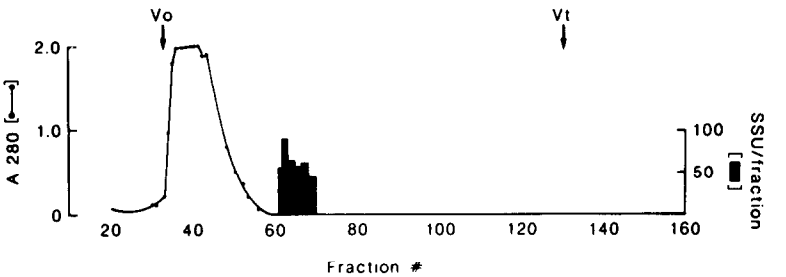

Figure 1: Acid gel filtration of 150 ml of plasma on a 10 x 100 cm Sephadex G-50 column. A standard somatomedin unit (SSU) is the amount of Sm-C/IGF-I measured by the RIA in 0.6 ml of the serum standard.

approximately 0.3-0.4 (Fig. 1). Analysis of the recovered material by SDS-PAGE revealed multiple bands of contaminating proteins with little staining at the expected location of Sm-C/IGF-I. (Fig. 2).

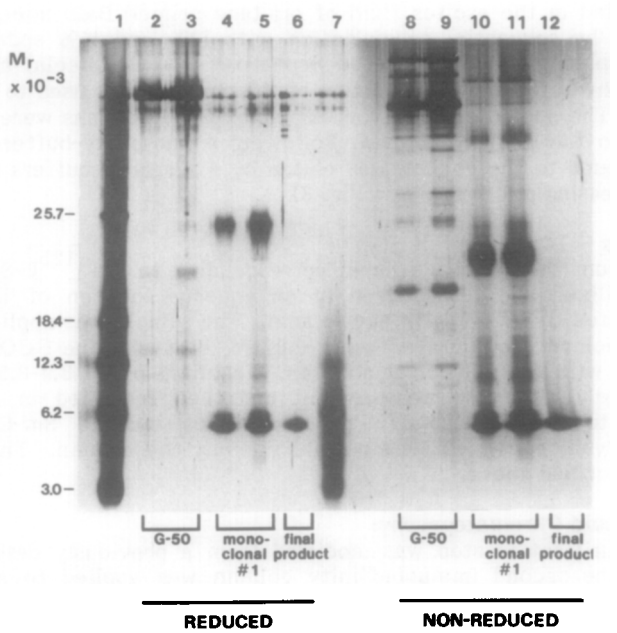

Figure 2: SDS-PAGE of purified Sm-C/IGF-I. The samples were analyzed on a 15% acrylamide gel according to the method of Laemli (15) and visualized using a sensitive silver stain method (14). Samples in lanes 1-7 were treated with 2-mercaptoethanol prior to electrophoresis whereas those in lane 8-12 were dissolved in gel sample solution containing no reducing agent. In lanes 2,3,8 and 9 an aliquot of Sephadex G-50 eluate was analyzed at 2 doses (1 µg protein lanes 2 and 8; 2 µg lanes 3 and 9) after lyophilization and dissolution in gel sample solution. Lanes 4,5,10 and 11 contain samples from the product of the initial immunoaffinity step of 144 ng of Sm-C, based on RIA, (lanes 4 and 10) and 228 ng (lanes 5 and 11). The final product after liquid chromatography (approximately 400 ng) was analyzed in lanes 6 and 12. Molecular weight standards used were: α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; cytochrome-C, 12,300; bovine lung trypsin inhibitor, 6,200; insulin, 3,000.

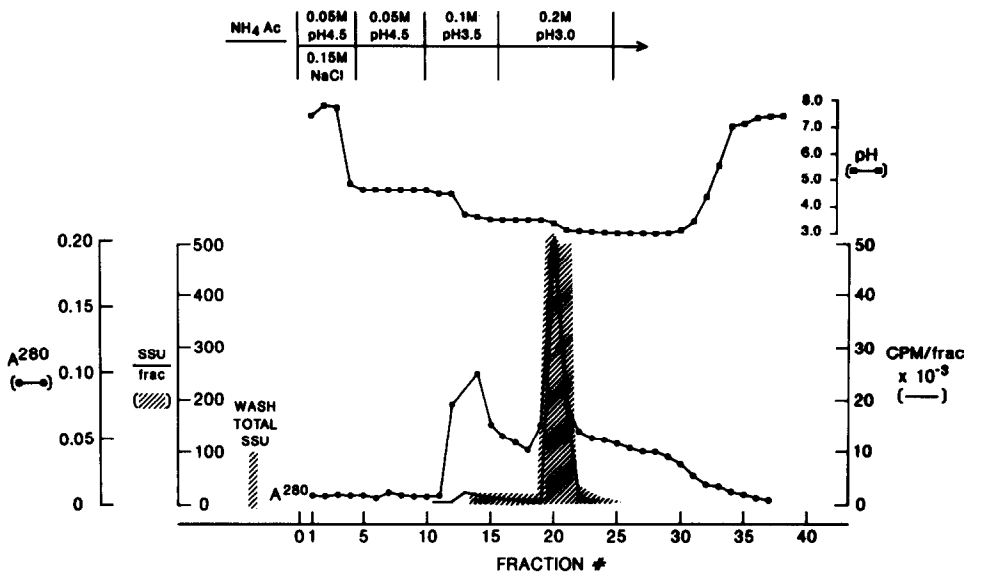

Figure 3: Elution of Sm-C/IGF-I from the monoclonal antibody column by a series of ammonium acetate buffers.

Affinity Chromatography:

The monoclonal antibody column extracted 90% of the Sm-C/IGF-I from the G-50 eluate (Fig. 3). Although the majority of contaminating proteins were not bound by the column during sample application, additional, weakly-bound contaminants were removed by washing with 0.1M ammonium acetate buffer (pH 3.5). Immunoreactive Sm-C/IGF-I and the [^{125}I]-Sm-C/IGF-I added to the sample prior to affinity chromatography co-eluted in the 0.2M ammonium acetate buffer (pH 3.0). This partially purified Sm-C/IGF-I appeared as a major band when analyzed on SDS-PAGE (Fig.2).

Chromatofocusing:

The majority of the Sm-C/IGF-I eluted in an irregular pattern between pH 8.0-8.5 along with the added [^{125}I]-Sm-C/IGF-I (Fig. 4). A small quantity of immunoreactive hormone appeared at pH 9.0 similar to the more basic somatomedin species that have been observed in other purification schemas (4,5).

Liquid Chromatography:

Immunoreactive Sm-C/IGF-I from the pH 8.0 - 8.5 chromatofocusing pool was separated from the polybuffers by using the affinity column and from the remaining contaminating proteins by reverse phase liquid chromatography. A single peak of Sm-

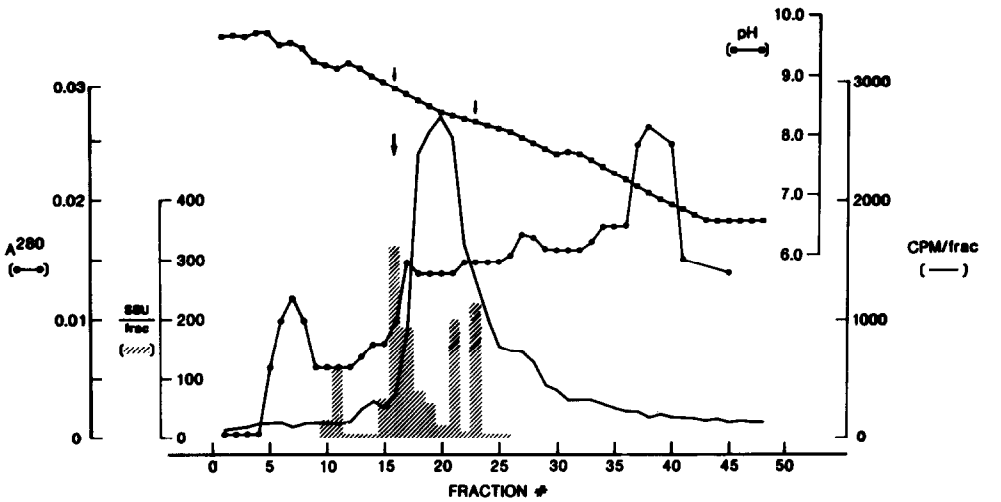

Figure 4: Results of chromatofocusing. Fractions between arrows were pooled for subsequent purification.

C/IGF-I eluted at approximately 40% acetonitrile. (Fig. 5). Under the conditions employed, [^{125}I]-Sm-C/IGF-I eluted slightly after the purified hormone (data not shown). When analyzed by SDS-PAGE, the purified product appeared as a single band migrating with the same mobility as a pure Sm-C/IGF-I standard both before and after disulfide bond reduction. The quantity of pure Sm-C/IGF-I recovered was 35 μg as measured by the RIA.

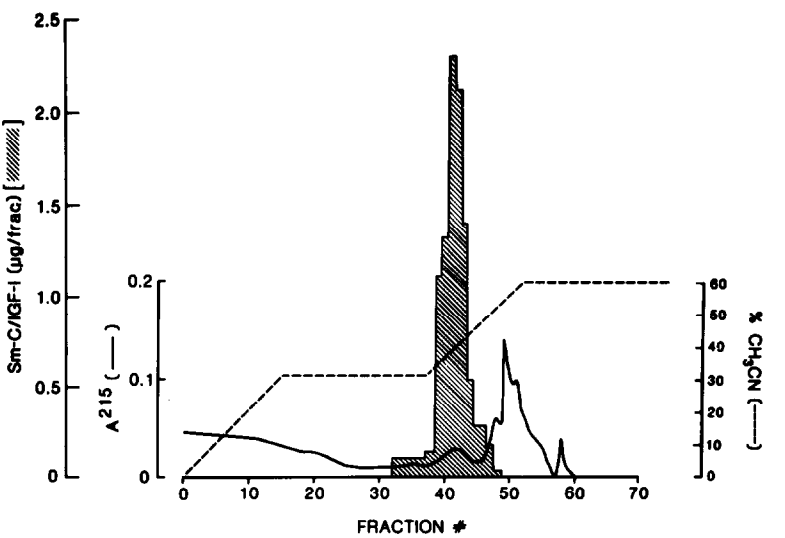

Figure 5: Results of reverse phase liquid chromatography. Hatched bars denote elution of Sm-C/IGF-I.

Evaluation of Purified Product:

When the purified hormone was iodinated using chloramine-T (2) and tested in the Sm-C/IGF-I RIA, 40% of the labeled hormone was bound at a 1:20,000 dilution of the rabbit anti-Sm-C/IGF-I antibody. Parallel studies with radiolabeled Sm-C/IGF-I isolated from Cohn-IV gave nearly identical results. The ability of the immunoaffinity-derived, labeled hormone to bind to the Sm-C/IGF-I receptor was demonstrated by affinity labeling human placental membranes with [^{125}I]-Sm-C/IGF-I using the non-specific cross-linking agent disuccinimidyl suberate as previously described (6). When the affinity labeled membranes were analyzed by SDS-PAGE and autoradiography after disulfide bond reduction, the predominantly labeled specific band had an apparent MW of 135,000 corresponding to the α subunit of the Sm-C/IGF-I receptor (data not shown). The pattern of labeling was the same whether the ^{125}I-Sm-C/IGF-I was derived from monoclonal antibody column purified hormone or from the standard preparation.

DISCUSSION:

The scarcity of pure somatomedin preparations continues to restrict research in somatomedin chemistry and physiology. Although active preparations of Sm-C have been chemically synthesized (7) and produced by recombinant DNA techniques (8), nearly all the pure somatomedin preparations available to investigators have been isolated by standard protein purification methodology from Cohn fraction IV, a by-product of the blood fractionation industry (4,5,9,10). Obtaining milligram quantities of pure somatomedins by these methods involves a laborious and costly purification accompanied by the potential exposure to hepatitis and other viruses frequently present in pooled plasma products.

Since monoclonal antibodies can easily be produced in large quantitites, high capacity immunoaffinity columns have been recently used to purify several proteins (11,12). The present study similarly demonstrates that rapid purification of Sm-C/IGF-I can be achieved with a monoclonal antibody affinity column. An analogous method has been proposed by Laubli et al. in a preliminary communication (13). In our method, Sm-C/IGF-I was first separated from its binding proteins by gel filtration and purified further by immunoaffinity chromatography. Chromatofocusing was then utilized to segregate remaining peptides on the basis of charge and remove any residual insulin-like growth

factor II retained by the affinity column. Somatomedin-C was ultimately purified by reverse phase liquid chromatography. The addition of [^{125}I]-Sm-C/IGF-I to the mixture during purification greatly facilitated the isolation by obviating the need for immunoassay prior to each step. Our overall yield of 18% was several fold better than the 2-5% achieved by the methods previously described for purification from Cohn fraction IV (4,5,9,10). The Sm-C/IGF-I purified by this method was homogeneous and behaved as a single peptide chain after disulfide bond reduction when analyzed using a sensitive silver stain after SDS-PAGE. Moreover, when iodinated, it reacted appropriately in the Sm-C/IGF-I RIA and could be covalently attached to the Sm-C/IGF-I receptor of human placental tissue. Thus, this relatively simple purification procedure yielded highly pure Sm-C/IGF-I suitable for receptor studies, RIA, and in vitro studies of biologic actions.

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